D-3-Hydroxybutyric acid

Colorimetric method

for the determination of D-3-hydroxybutyric acid in foodstuffs and other materials

Cat. No. 0 907 979

Test-Combination for 3 × approx. 12 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For use in *in vitro* only

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

In the presence of the enzyme 3-hydroxybutyrate dehydrogenase (3-HBDH), D-3-hydroxybutyric acid (D-3-hydroxybutyrate) is oxidized by nicotinamide-adenine dinucleotide (NAD) to acetoacetate (1).

(1) D-3-Hydroxybutyrate + NAD^+ $\xrightarrow{\text{3-HBDH}}$ acetoacetate + NADH + H^+

In the reaction catalyzed by diaphorase the NADH formed converts iodonitrotetrazolium chloride (INT) to a formazan which is measured at its maximum in the visible range at 492 nm (2).

(2) NADH + INT + H^+ $\xrightarrow{\text{diaphorase}}$ NAD^+ + formazan

The equilibrium of reaction (1) is displaced quantitatively in favour of acetoacetate by trapping the formed NADH by INT.

The Test-Combination contains

1. Bottle 1 with approx. 25 ml solution, consisting of: potassium phosphate/triethanolamine buffer, pH approx. 8.6; Triton X-100 (trademark of Rohm & Haas, Philadelphia, USA)
2. Three bottles 2 with each approx. 35 mg lyophilizate, consisting of: diaphorase, approx. 4 U; NAD, approx. 28 mg
3. Bottle 3 with iodonitrotetrazolium chloride solution, approx. 2.5 ml
4. Bottle 4 with approx 1.8 ml 3-hydroxybutyrate dehydrogenase suspension, approx. 27 U

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve contents of one bottle 2 in 2.5 ml of redist. water.
3. Dilute contents of bottle 3 with 6 ml redist. water.
4. Use contents of bottle 4 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).
Bring solution 1 to 20-25°C before use.
The contents of the bottles 2 are stable at 2-8°C (see pack label).
Solution 2 is stable for 1 week at 2-8°C.
Bring solution 2 to 20-25°C before use.
The contents of bottle 3 are stable at 2-8°C (see pack label).
Solution 3 is stable for 3 months at 2-8°C and for 1 month at 20-25°C stored in the dark.
Bring solution 3 to 20-25°C before use.
The contents of bottle 4 are stable at 2-8°C (see pack label).

Procedure

Wavelength: (Hg) 492 nm
Glass cuvette[1]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.050 ml
Read against air (without a cuvette in the light path) or against water or blank
Sample solution: 0.4-12 µg D-3-hydroxybutyrate/assay[2] in 0.100 - 2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1[3]	0.600 ml	0.600 ml
solution 2[3]	0.200 ml	0.200 ml
solution 3[3, 4]	0.200 ml	0.200 ml
sample solution*	-	0.100 ml
redist. water	2.000 ml	1.900 ml
Mix**, after 2 min read absorbances of the solutions (A_1). Repeat the measurement after 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be pre-treated according to pt. 7.2 (removal of reducing substances). However, if the change in absorbance is less than 0.010, such pre-treatment is not necessary, providing the reaction is started immediately after the previous measurement by addition of:		
suspension 4	0.050 ml	0.050 ml
Mix**, read absorbances of the solutions **exactly 20 min** after the addition of suspension 4 (A_2). Repeat the reading after **exactly 10 min** (A_3).		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

Absorbance difference for the blank:

$\Delta A_{blank} = (A_2\text{-}A_1)_{blank} - 2 \times (A_3\text{-}A_2)_{blank}$

Absorbance difference for the sample:

$\Delta A_{sample} = (A_2\text{-}A_1)_{sample} - 2 \times (A_3\text{-}A_2)_{sample}$

Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = \Delta A_{sample} - \Delta A_{blank}$$

For acceleration of the measurement and for simplification of the calculation, the measurement of A_3 can be neglected with less accuracy of the result. In this case calculate the absorbance difference (A_2-A_1) for blank and sample.

Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of formazan at 492 nm
= 19.9 [l × $mmol^{-1}$ × cm^{-1}]

It follows for D-3-hydroxybutyric acid:

$$c = \frac{3.050 \times 104.1}{19.9 \times 1.00 \times 0.100 \times 1000} \times \Delta A = 0.1596 \times \Delta A$$ [g D-3-hydroxybutyric acid/l sample solution]

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

1 If desired disposable cuvettes may be used instead of glass cuvettes.
2 See instructions for performance of assay
3 For series analysis, a suitable stock solution may be prepared by mixing solutions 1, 2 and 3. This stock solution is stable for 1 h when stored in the dark at 20-25°C. Use 1.000 ml for analysis.
4 **INT is sensitive to light. After addition of solution 3, do not store the cuvette in the light.**

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$$\text{Content}_{\text{D-3-hydroxybutyric acid}} = \frac{c_{\text{D-3-hydroxybutyric acid}}[\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 1000\ [\text{g/1000 g}]$$

1. Instructions for performance of assay

The amount of D-3-hydroxybutyric acid present in the assay has to be between 0.4 µg and 12 µg. In order to get a sufficient absorbance difference, the sample solution is diluted until a D-3-hydroxybutyric acid concentration of not more than 0.12 g/l is obtained.

Dilution table

Estimated amount of D-3-hydroxybutyric acid/l	Dilution with water	Dilution factor F
< 0.12 g	–	1
0.12-1.2 g	1 + 9	10
1.2-12 g	1 + 99	100
> 12 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

2.1 For series analysis, a suitable stock solution may be prepared by mixing solutions 1, 2 and 3. This stock solution is stable for 1 h when stored in the dark. Use 1.000 ml for each assay.

2.2 The reaction system is sensitive to light (daylight or artificial light) after the addition of INT (solution 3 or reaction mixture). The incubation has to be done in the dark:

a) if incubating in the photometer, close cuvette compartment of the photometer and block out light.

b) Cover the cuvettes or store in a darkened cupboard.

2.3 Sample preparation with concentrated Carrez-solutions has proved beneficial in the analysis of liquid whole egg and whole egg powder (see ref. 2.1). The sample solution can also be used for the determination of L-lactic acid and of succinic acid.

2.4 In carrying out the calculation, the results are usually given as D-3-hydroxybutyric acid (molar mass 104.1 g/mol). (In enzymatic determinations, the D-3-hydroxybutyrate ion is measured.)

2.5 Free hydroxybutyric acid cannot be used for the preparation of assay control solutions because the reaction is not a quantitative one. The reason for this may be (similar to lactic acid) the formation of esters which are not converted in the enzymatic reaction. It is recommended to use sodium D,L-3-hydroxybutyrate for the preparation of assay control solutions.

3. Specificity (Ref. 1)

The method is specific for D-3-hydroxybutyric acid.

In the analysis of commercial mono-sodium-D,L-hydroxybutyrate (molecular weight 126.1), results of approx. 50% have to be expected. (Only the D-form is measured enzymatically.)

4. Sensitivity and detection limit

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml of a D-3-hydroxybutyric acid concentration of 0.04 mg/l sample solution (if v = 0.100 ml, this corresponds to 0.8 mg/l sample solution).

The detection limit of 0.2 mg/l is derived from the absorbance difference of 0.020 and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from 0.4 µg D-3-hydroxybutyric acid/assay (0.2 mg D-3-hydroxybutyric acid/l sample solution; sample volume v = 2.000 ml) to 12 µg D-3-hydroxybutyric acid/assay (0.12 g D-3-hydroxybutyric acid/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a D-3-hydroxybutyric acid concentration of approx. 0.8 - 1.5 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of approx. 0.01-0.015 g/100 g can be expected.)

The following data have been published in the literature:

Whole egg powder:

x = 4 mg/kg	r = 3.0 mg/kg	$s_{(r)} = \pm$ 1.1 mg/kg	
	R = 4.1 mg/kg	$s_{(R)} = \pm$ 1.5 mg/kg	(Ref. 2.1)

7. Interference/sources of error

7.1 Test-Combinations which are used after the stated date of expiration, or reagent mixtures of diaphorase, NAD, and INT stored for more than one hour at 20-25°C (see[3]) before being used give rise to a delayed reaction or produce a creep reaction which must be taken into account in the calculation of A_2 by extrapolation to the time of addition of suspension 4 (3-HBDH).

7.2 High concentrations of reducing substances, e.g. L-ascorbic acid or sulfurous acid, interfere in the assay because of their reaction with INT. This interference is eliminated by preceding treatment of the sample with H_2O_2:

Weigh the sample or pipette the sample solution respectively, diluted if necessary, into a 50 ml volumetric flask. Fill up with water to a volume of about 40 ml, add 0.5 ml KOH (2 M) and 0.01 ml H_2O_2 (30% w/v), incubate for 10 min at approx. 70°C. Adjust to pH 7-8 with H_2SO_4 (1 M). Allow the solution to cool to 20-25°C. Fill up to the mark with water; mix the solution, filter, if necessary. Use the filtrate for the assay.

8. Recognizing interference during the assay procedure

8.1 If the conversion of D-3-hydroxybutyric acid has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding mono-sodium D,L-3-hydroxybutyrate (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of D-3-hydroxybutyric acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to pH 8 by adding sodium or potassium hydroxide solution;
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary;
Deproteinize **samples containing protein** with perchloric acid; alternatively clarify with Carrez reagents;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter; alternatively clarify with Carrez-solutions after the extraction with hot water.

Carrez clarification:
Pipette the liquid sample into a 100 ml volumetric flask containing approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4 \times 7\ H_2O$/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

Preparation of egg and egg product samples is dealt with in pt. 11 (application examples). Note: Treatment with concentrated Carrez-solutions has proved beneficial in routine analysis. In Germany, the method has been standardized and published in the official methods collection acc. to § 35 of the Foodstuffs and Consumer Goods Law (Lebensmittel- and Bedarfsgegenständegesetz, LMBG). The sample solution resulting from Carrez clarification can also be used for the determination of L-lactic acid and of succinic acid.

11. Application examples

Determination of D-3-hydroxybutyric acid in liquid whole egg
a) Sample preparation with Carrez clarification (Ref. 2.1)
Accurately weigh approx. 5 g homogeneous liquid whole egg into a 25 ml volumetric flask, add 10 ml redist. water and one drop n-octanol, mix and incubate in a boiling water-bath for 15 min. Allow to cool to 20-25°C. For clarification, add one after the other and shake after each addition: 1 ml concentrated Carrez-I-solution (15.0 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml), 1 ml concentrated Carrez-II-solution (30 g zinc sulfate, $ZnSO_4 \times 7\ H_2O$/100 ml). Fill up to the mark with sodium hydroxide (0.1 M), mix and filter with fluted filter paper and glass funnel. Use the filtrate for the assay (v = 0.100 ml in the case of incubated fertilized eggs, v = 1.000 ml in the case of fresh eggs).

The concentration of the sample in the sample solution (c_{sample}) is approx. 5 g/25 ml, corresponding to approx. 200 g/liter.

Recovery experiments
For checking sample preparation and assay, recovery experiments may be carried out. A solution of D,L-3-hydroxybutyrate, mono-sodium salt is prepared: 0.4 g/100 ml for the analysis of incubated fertilized eggs (v = 0.100 ml) and 0.4 g/l for the analysis of fresh eggs (v = 1.000 ml).

1.000 ml of this solution is added to the homogeneous liquid whole egg weighed out for sample preparation with Carrez clarification. The volume of water added must then be reduced correspondingly (9 ml instead of 10 ml).

b) Sample preparation with perchloric acid deproteinization
Accurately weigh approx. 20 g (a) homogeneous liquid whole egg into a 100 ml beaker. Add 20 ml (b) of a perchloric acid solution (prepared from 40 volumes perchloric acid (60%, w/v) and 60 volumes redist. water), homogenize with an Ultra-Turrax for approx. 5 min and filter. 10 ml filtrate (d) are adjusted with KOH (30%, w/v) at the beginning and with KOH (1 M) at the end to pH 8.4 (7.8-8.6) on a pH-meter. Measure the volume of KOH used for pH adjustment (e). Place the solution in an ice-bath for 15 min and filter the cold material. Use the clear filtrate, brought to 20-25°C, for the assay (v = 0.100 ml in the case of incubated fertilized eggs).

For calculating the D-3-hydroxybutyric acid content in liquid whole egg according to the above mentioned formula (see calculation) the weight of the sample in the sample solution is to be first calculated according to the following formula:

$$\text{Weight}_{sample} = \frac{a \times 1000 \times d}{(b + a \times 0.75) \times (d + e)}\ [g/l]$$

It is:
a: the weighed liquid whole egg in g
1000: factor for g expressed in mg
d: volume of filtrate in ml
b: volume of perchloric acid in ml
0.75: water content of liquid whole egg in (%;w/w)/100
e: volume of KOH in ml

(The specific gravity of water from the sample at 20-25°C is approx. 1 g/ml. It can be neglected for the calculation.)

Recovery experiment
For checking sample preparation and assay, recovery experiments may be carried out. A solution of D,L-3-hydroxybutyrate, monosodium salt is prepared: 0.4 g/100 ml for the analysis of incubated fertilized eggs (v = 0.100 ml) and 0.4 g/l for the analysis of fresh eggs (v = 1.000 ml).

2.000 ml of this solution are added to the homogeneous liquid whole egg weighed out for sample preparation with perchloric acid deproteinization. The volumes of perchloric acid to be added have to be reduced correspondingly (18 ml instead of 20 ml) or the changed volumes have to be taken into account when calculating the results.

Interference
Occasionally it may be observed that, e.g. after deproteinization with perchloric acid, the absorbance A_1 is not constant. In that case it is recommended to run a sample blank (assay like "sample", but without suspension 4). The absorbance difference $(A_2\text{-}A_1)_{sample\ blank}$ has to be subtracted from ΔA.

c) Extraction of heat-coagulated egg
Homogenize the contents of an egg for 5 min. Weigh 2 g of the homogeneous material (accuracy 1 mg) into a 100 ml Erlenmeyer flask and cover the flask with a glass. Heat the flask in a water-bath at 85°C for 45 min to inactivate the egg enzymes. Allow to cool to 20-25°C, remove the solid coagulated egg from the bottom of the flask, add 8 ml of dist. water and mix rigorously for 10 min with a magnetic stirrer. Filter through fluted filter paper and use the slightly turbid filtrate for the assay (v = 0.100 ml in the case of incubated fertilized eggs, v = 1.000 ml in the case of fresh eggs.

The altered sample volume (v = 1.000 ml) must be taken into account in the calculation.

The measured absorbance difference (ΔA) should not exceed 0.400, otherwise the results would be too low.

When calculating the concentration of D-3-hydroxybutyric acid in egg (in g/1000 g) the result must be multiplied by the factor F = 4.08. This factor results from the volume of water used for sample preparation (8.0 ml) and the "free" water that can be extracted from the egg material (0.16 ml), based on 2 g of egg. This factor is proved by recovery experiments. F = (8.0 + 0.16):2.0.

Determination of D-3-hydroxybutyric acid in egg products
Adjust liquid egg sample (homogeneous whole egg, salted whole egg, yolk, white of egg.) to 20-25°C and homogenize carefully: Weigh 2 g of the sample into a 100 ml-Erlenmeyer flask and treat as mentioned under "liquid whole egg".

Determination of D-3-hydroxybutyric acid in whole egg powder (Ref. 2.1)
Accurately weigh approx. 1 g whole egg powder into a 25 ml volumetric flask, add 12 ml redist. water and one drop n-octanol, mix and heat in a water-bath (approx. 100°C) for 15 min. Allow volumetric flask to cool to 20-25°C, add one after the other and shake rigorously after each addition: 1 ml concentrated Carrez-I-solution (15.0 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml), 1 ml concentrated Carrez-II-solution (30.0 g zink sulfate, $ZnSO_4 \times 7\ H_2O$/100 ml). Adjust pH to approx. 8-9 with sodium hydroxide (1 M), fill up to the mark with redist. water, mix and filter with fluted filter paper and glass funnel. Use 0.100-2.000 ml filtrate for the assay. Take the altered sample volume into account in the calculation.

Determination of D-3-hydroxybutyric acid in protein-containing samples
Homogenize 15 g or 15 ml of the homogeneous sample with 15 ml perchloric acid (1 M) for 10 min (using a homogenizer, or Ultra Turrax). Centrifuge and decant the supernatant. Adjust the supernatant to pH 7-8 by the addition of solid $KHCO_3$, if necessary add 1 drop of n-octanol to prevent foaming. To obtain quantitative precipitation of the potassium perchlorate formed, place in an ice-bath or refrigerator for 20 min and filter. Adjust the filtrate to 20-25°C and use for the assay, dilute according to the dilution table, if necessary.
When calculating the dilution, take the water content of the sample into account.

12. Further applications

The method may also be used in research for the analysis of biological samples. For details of sampling, treatment and stability of the sample see Ref. 1.2 and 1.3.

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D-3-Hydroxybutyric acid assay control solution

The assay control solution serves as a control for the enzymatic determination of D-3-hydroxybutyric acid in foodstuffs and other materials.

Reagents

D,L-3-Hydroxybutyric acid, mono-sodium salt

Preparation of the assay control solution

Accurately weigh approx. 30 mg mono-sodium D,L-3-hydroxybutyrate to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly (this corresponds approx. 0.12 g D-3-hydroxybutyric acid/l).

Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

Application:

1. Addition of D,L-3-hydroxybutyric acid assay control solution to the assay mixture:

Instead of sample solution the assay control solution is used for the assay. (It has to be considered when calculating results that in the enzymatic determination only the D-form is measured.)

(The measurement of the assay control solution is not necessary for calculating results.)

2. Restart of reaction, quantitatively:

After completion of the reaction with sample solution and measuring A_3, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_4 after the end of the reaction (approx. 30 min). Calculate the concentration from the difference (A_4 -A_3) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by the addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. Internal standard:

The assay control solution can be used as internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 1	0.600 ml	0.600 ml	0.600 ml	0.600 ml
solution 2	0.200 ml	0.200 ml	0.200 ml	0.200 ml
solution 3	0.200 ml	0.200 ml	0.200 ml	0.200 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
Mix, and read absorbances of the solutions (A_1) after approx. 2 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

4. Recovery experiments with original samples:

For checking sample preparation and assay, recovery experiments may be carried out. For this, either the a. m. assay control solution is used or another assay control solution with a suitable concentration is prepared.

The original sample is measured with and without added D-3-hydroxybutyric acid. The amount of added D-3-hydroxybutyrate

- is either the same as expected to be present in the original sample,
- or corresponds that amount of D-3-hydroxybutyrate which is allowed to be contained in the sample e.g. according to standards or other regulations.

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